

Biophysical Characterization of Styryl Dye-Membrane Interactions

Yao Wu,^{†‡} Felix L. Yeh,^{†‡} Fei Mao,[§] and Edwin R. Chapman^{†‡*}

[†]Howard Hughes Medical Institute and [‡]Department of Physiology, University of Wisconsin, Madison, Wisconsin; and [§]Biotium, Inc., Hayward, California

ABSTRACT Styryl dyes (also referred to as FM dyes) become highly fluorescent upon binding to membranes and are often used to study synaptic vesicle recycling in neurons. To date, however, no direct comparisons of the fluorescent properties, or time-resolved (millisecond) measurements of dye-membrane binding and unbinding reactions, for all members of this family of probes have been reported. Here, we compare the fluorescence intensities of each member of the FM dye family when bound to membranes. This analysis included SGC5, a new lipophilic fluorescent dye with a unique structure. Fluorescence intensities depended on the length of the lipophilic tail of each dye, with a rank order as follows: SGC5 > FM1-84 > FM1-43 > Synapto-Green C3 > FM2-10/FM4-64/FM5-95. Stopped-flow measurements revealed that dye hydrophobicity determined the affinity and partitioning rates for dye-membrane interactions. All of the dyes dissociated from membranes on the millisecond timescale, which is orders of magnitude faster than the overall destaining rate (timescale of seconds) of these dyes from presynaptic boutons. Partitioning kinetics were faster at higher temperatures, but were unaffected by pH or cholesterol. The data reported here aid interpretation of dye-release kinetics from single synaptic vesicles, and indicate that these probes dissociate from membranes on more rapid timescales than previously appreciated.

INTRODUCTION

Pioneering studies by Katz and co-workers (1) established that presynaptic nerve terminals release neurotransmitters in discrete quanta. Subsequent studies further confirmed that synaptic vesicles (SVs), loaded with neurotransmitters, give rise to these quanta by fusing with the presynaptic plasma membrane (2–4). After exocytosis, SVs recycle from the plasma membrane by endocytosis and are reloaded with transmitter for future rounds of transmitter release. Until recently, the synaptic vesicle cycle was largely studied via postsynaptic electrophysiological recordings or by ultrastructural analysis of fixed samples. Direct measurements of the synaptic vesicle cycle in intact neurons required the development of new tools to study this process. This need was met in 1992 when Betz et al. (5) characterized a styryl dye—FM1-43—and effectively used it to study the synaptic vesicle cycle in the intact neuromuscular junction of frogs. Since the publication of that groundbreaking study, numerous dyes with slightly different properties have been generated and used to study SV trafficking and to address the idea that there are distinct modes of exocytosis in at least some central synapses (6–11).

In general terms, exocytosis proceeds through a crucial intermediate termed the fusion pore, in which a transient aqueous connection is formed between the vesicle lumen and the extracellular space. It has been proposed that once

the fusion pore opens, it has at least two choices, each of which has distinct physiological ramifications (8,12–16). During the well-established full-fusion pathway, the pore always dilates after opening, resulting in the collapse of the vesicle into the plasma membrane (2,17). In the second putative mode—often referred to as “kiss-and-run” exocytosis—the fusion pore is thought to undergo a reversal from the open state back to the closed state, which could occur without the complete merger of the vesicle and plasma membrane (18–22). During kiss-and-run exocytosis, the small size of the fusion pore could limit the rate of secretion to potentially drive desensitization (e.g., of AMPA receptors), rather than activation. Moreover, a small fusion pore could act as a “size exclusion filter”, allowing the preferential escape of smaller hormones (e.g., in neuroendocrine cells) while retaining larger hormones that can only be released upon full fusion (23). Kiss-and-run events have been detected via capacitance measurements in the posterior pituitary, chromaffin cells, mast cells, eosinophils, neutrophils, and PC12 cells (15,20,21,24–28), and by amperometric measurements and optical approaches in endocrine and neuroendocrine cells and cell lines (16,21,29–33). However, whether kiss-and-run occurs during synaptic vesicle exocytosis in neurons is a subject of debate (6,10,34–37).

Recent studies have used FM dyes in an attempt to discern between different modes of exocytosis in neurons (6–10). However, interpretation of FM dye destaining experiments requires a detailed understanding of the biophysical properties of the dyes themselves. Which dyes are bright enough to study release from single vesicles? Do the dyes partition from membranes with kinetics that are distinct enough from one another to allow differential destaining measurements?

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*Correspondence: chapman@physiology.wisc.edu

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(Full fusion collapse should result in rapid and complete loss of all dyes, whereas kiss-and-run should preferentially retain “slower” dyes.) What are the timescales for departitioning versus the open time of a putative kiss-and-run fusion pore?

Earlier studies in which dye-unbinding kinetics were measured via perfusion of cultured neurons have not resulted in a consensus regarding dye kinetics, and rapid time-resolved methods have been applied to only a single dye in the family (FM1-43) (7,38). Therefore, we carried out detailed biophysical studies of all members of the FM dye family, along with a new dye: SGC5. We measured the relative brightness of these dyes and their affinities for membranes, and determined the rates at which they bind and unbind from membranes.

MATERIALS AND METHODS

Dyes

FM1-84, FM1-43, FM2-10, FM4-64, and FM5-95 were obtained from Invitrogen (Carlsbad, CA). SynaptoGreen C3 and SGC5 were synthesized by F. Mao (Biotium, Hayward, CA).

Liposomes

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC), total brain lipid extract, and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). Lipids were dissolved in chloroform, dried under nitrogen, and vacuum-lyophilized for 1 h. Dried lipid films were then resuspended in HEPES buffer (50 mM HEPES and 100 mM NaCl, pH 7.4). Liposomes composed of total brain lipid were prepared by sonication (~50 nm) using a Microson ultrasonic cell disruptor (Misonix, Farmingdale, NY), and 100 nm liposomes composed of 30% PE/70% PC and additional 0%, 5%, 15%, and 45% cholesterol were prepared by extrusion (7,39).

Steady-state fluorescence measurements

Steady-state fluorescence measurements were performed using a QM-1 spectrophotometer (PTI, South Brunswick, NJ) at 22°C. Dyes were mixed with liposomes using a castle-style stir bar in a cuvette.

Spectra of dyes after loading into hippocampal neurons

Hippocampal neurons were cultured as previously described (7,40), and 14- to 21-day-old neurons were loaded with dyes (FM1-84 and SGC5, 2 μ M; FM1-43 and FM4-64, 10 μ M; SynaptoGreen C3, 20 μ M; FM2-10 and FM5-95, 40 μ M) in modified Tyrode solution (99 mM NaCl, 55 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4) for 3 min at room temperature. Neurons were then washed in calcium-free Tyrode solution (150 mM NaCl, 4 mM KCl, 4 mM MgCl₂, 0 mM CaCl₂, with all other components kept unchanged) for 3–10 min. Dye spectra were obtained using an Olympus FV1000 confocal upright microscope (Olympus, Melville, NY) with a 488 nm laser to excite the dyes, and neurons were imaged with a 60 \times LUMFL 1.10 water immersion objective (Olympus). A 405/488 dichroic mirror was used and the emission spectra were collected from 500 nm to 785 nm at 15 nm intervals. Data were collected and analyzed using the Olympus Fluoview 1.7a software.

Stopped-flow rapid mixing experiments

Kinetic experiments were performed using an Applied Photophysics SX 18 MV stopped-flow spectrometer (Leatherhead, Surrey, UK). Emitted light was collected through a 530 nm cutoff filter. For association experiments, dyes and liposomes were loaded into two different syringes and rapidly mixed (dead time ~1 ms). The final dye concentration was 4 μ M. For dissociation experiments, 4 μ M dye bound to membrane (2.2 nM liposomes) was diluted 1:11 with HEPES buffer. The on- (k_{on}) and off- (k_{off}) rates were calculated assuming pseudo first-order kinetics according to the following equation: $k_{\text{obs}} = [\text{liposome}] k_{\text{on}} + k_{\text{off}}$ (7,38) (see Fig. 5, C–I). For departitioning experiments, the rapid fluorescence changes were well fitted by single or, in some cases, double (e.g., FM1-84 and FM1-43) exponential functions. The reason for the second slow component is unclear, but it may involve the presence of impurities in the dye preparations; this represented a minor component (~10%) of the fluorescence signals. For these reasons, and to simplify comparisons among the different dyes examined, we fit all of the data with single exponential functions.

RESULTS

Quantitative comparison of the fluorescence changes exhibited by FM dyes and SGC5 upon binding to membranes

There are six members of the FM dye family, and their structures are shown in Fig. 1. The lipophilic “tails” of these dyes facilitate association with membranes, resulting in a marked increase in fluorescence intensity of the fluorophore core that lies in the middle of each FM dye structure. The spectral properties of these dyes are determined by the two aromatic rings and the connecting double bond in the core region; increasing the number of double bonds in the bridge results in a red shift in the emission spectra (e.g., FM 4-64 and FM 5-95). The other end of the styryl dyes has a charged “head” that prevents complete insertion into membranes (41).

FM1-84, FM1-43, SynaptoGreen C3, and FM2-10 share an identical fluorophore core. The only difference among this series of dyes lies in the length of their lipophilic tails, and these differences are likely to give rise to different affinities and kinetics for binding to membranes. In this study, we addressed this issue by comparing the biophysical properties of all six members of the FM dye family in a side-by-side and quantitative manner, for the first time to our knowledge. In addition, we characterized another new lipophilic fluorescent dye, SGC5 (Fig. 1), which does not belong to the FM dye family but has similar fluorescence properties (Figs. 2 and 4).

As a first step in determining the fluorescence intensity and optimal concentration of the dyes to be used in membrane-binding experiments, we titrated each dye against a constant concentration of liposomes (2.2 nM liposomes composed of 30% PE/70% PC; Fig. 2, B–H). These titrations revealed that FM1-84 was the brightest FM dye, followed by FM1-43 and SynaptoGreen C3. FM2-10, FM4-64, and FM 5-95 were considerably dimmer (Fig. 2 I). These results are consistent with the prediction that an increase in the lipophilic tail length of a dye will result in more robust interactions with membranes (i.e., higher-affinity binding, deeper insertion into bilayers, or both) and hence greater increases

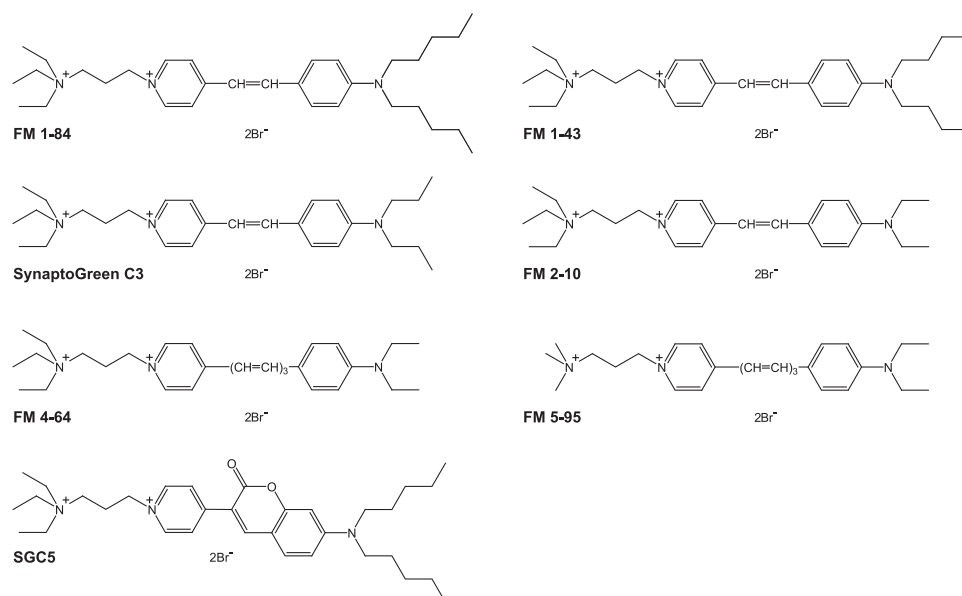


FIGURE 1 Dye structures. FM1-84, FM1-43, SynaptoGreen C3, and FM2-10 share identical fluorophore cores. All of the FM dyes have two lipophilic “tails”; in FM1-84, the lipophilic tails are composed of four methylene groups and a terminal methyl group. FM1-43, SynaptoGreen C3, and FM 2-10 have hydrocarbon tails composed of four, three, and two carbons, respectively. FM4-64 and FM5-95 have the same two-carbon tails, but have distinct charged “head” regions.

in fluorescence intensity. The novel dye, SGC5, also exhibited strong fluorescence increases upon binding to membranes (Fig. 2, *H* and *I*) and hence should be useful for studying synaptic vesicle recycling in neurons as well. SGC5 was therefore included in all experiments to examine the kinetics of dye-membrane interactions.

To determine the optimal concentrations of FM dyes for use in cell-based uptake experiments, we used the dye titration data (Fig. 2, *B–H*) to calculate the fold enhancement of the fluorescence intensity of each dye upon addition of liposomes (Fig. 2 *J*). FM1-84 exhibited the largest-fold increase upon membrane binding, followed by SGC5 and FM1-43.

To gain a more detailed understanding of the relative intensities and affinities of each dye for membranes, we next titrated increasing [liposome] against a fixed [dye]. FM1-84, FM1-43, and SGC5 exhibited the highest fluorescence intensities, whereas membrane-bound FM2-10, FM4-64, and FM5-95 were, again, relatively dim (Fig. 3 *A*). To facilitate comparisons among the different dyes, we plotted the fold enhancement of the fluorescence intensity of each dye as a function of increasing [liposome]. FM1-84, FM1-43, and SGC5 exhibited the largest-fold increase upon binding to membranes (Fig. 3 *B*). This is consistent with the findings reported in Fig. 2 *J*, where the dyes were titrated onto a fixed concentration of liposomes.

Since the fluorescence intensity directly reflects the [dye-membrane], the plot shown in Fig. 3 *A* can be used to approximate the relative affinity of the four dyes that share identical fluorophore cores. The rank affinities were FM1-84 > FM1-43 > SynaptoGreen C3 > FM2-10, and hence coincide with the length of the lipophilic tails in each dye.

To facilitate the utilization/application of the dyes in living cells, we loaded them into recycling synaptic vesicles in cultured hippocampal neurons and determined their emission spectra (Fig. 4 *A*). The emission spectra of FM1-84, FM1-43,

SynaptoGreen C3, and FM2-10 were almost identical to one another, with peak emission wavelengths at ~560 nm. The emission spectra of FM4-64 and FM5-95 were also similar to one another, with red-shifted emission peaks (relative to all the other dyes) at ~635 nm. Finally, the emission peak of SGC5 was ~530 nm (Fig. 4 *A*). There was no significant fluorescence emission beyond 700 nm for any of the dyes tested. In comparison with the spectra of dyes bound to 30% PE/70% PC liposomes (as well as the spectra provided by the vendor), the emission spectra of dyes loaded in cultured hippocampal neurons were all slightly blue-shifted (Fig. 4 *B*), which is likely due to differences in the local environment of the fluorophore cores of each dye (e.g., differences in membrane composition, the presence or absence of proteins, etc.).

Kinetics of dye-membrane interactions: effects of temperature, pH, and lipid composition

To determine the kinetics of FM dye-membrane binding and unbinding reactions, we performed stopped-flow rapid mixing experiments (Figs. 5 *A* and 6 *A*). For association experiments, dyes were kept constant at 4 μM , and the [liposome] (30% PE/70% PC) was varied from 1.1 to 5.5 nM (Fig. 5, *C* and *D*) or 3.3 to 7.7 nM (Fig. 5, *E–I*). Upon mixing, a rapid increase in fluorescence intensity (due to the formation of dye-membrane complexes) was observed (Fig. 5, *A* and *B*). The observed rate constants, k_{obs} , were calculated by fitting the fluorescence traces with single exponential functions (Fig. 5 *B*) and are plotted versus [liposome] in Fig. 5, *C–I*. The rate constants, k_{on} and k_{off} , were obtained from these plots as described in Materials and Methods, and these data are shown in Table 1. The resultant dissociation constants (K_{d} ; calculated from $k_{\text{off}}/k_{\text{on}}$) indicate that the order of affinity (from high to low) was as follows: FM1-84,

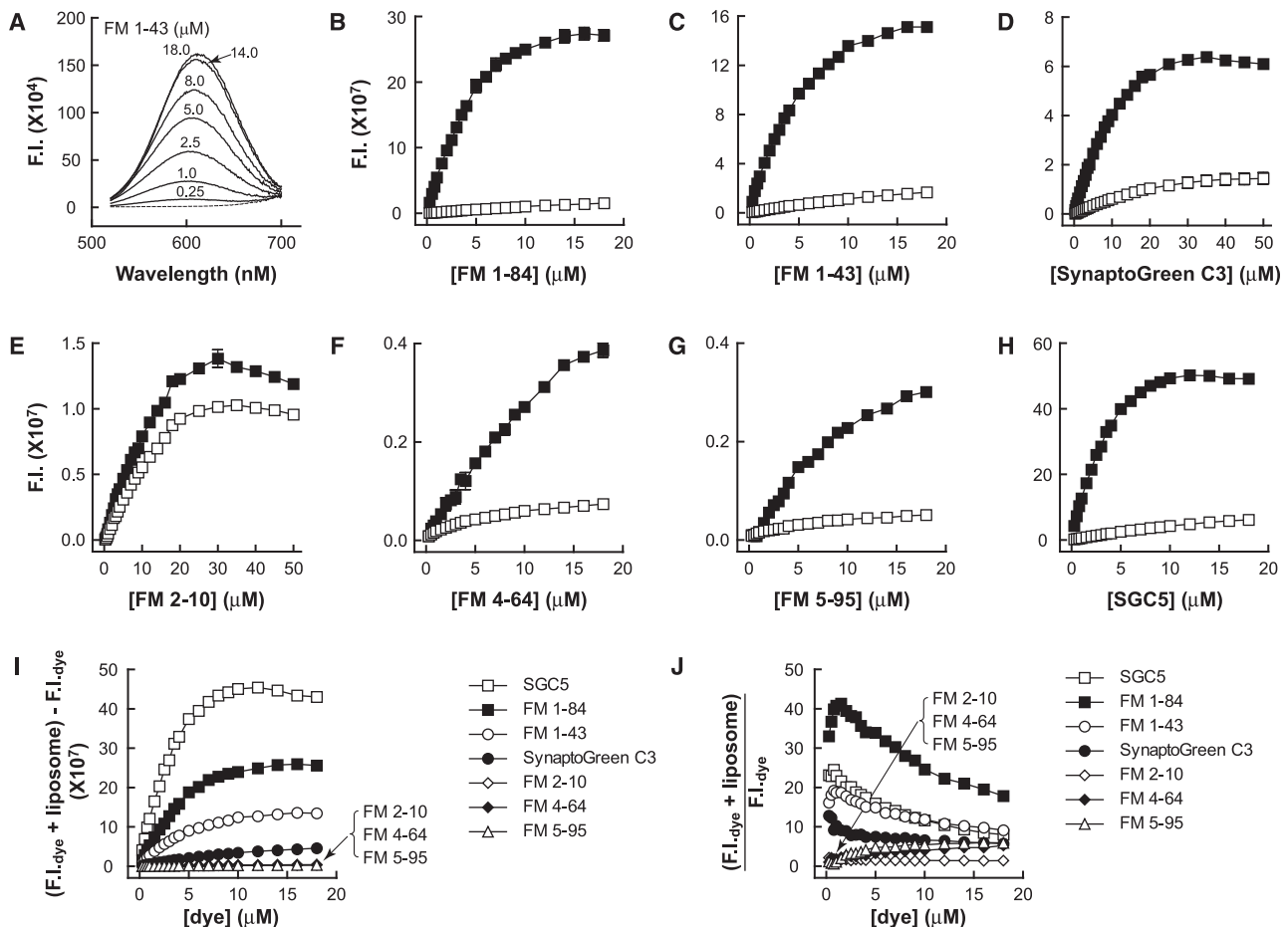


FIGURE 2 Fluorescence intensities of dye-membrane complexes as a function of increasing dye concentration. (A) Titration of liposomes (2.2 nM liposomes composed of 30% PE/70% PC) against [FM1-43]. The dashed line represents the signal obtained from liposomes only, and the solid lines are liposomes plus the indicated [FM1-43]. (B–H) Fluorescence intensities determined by integration of emission spectra of dye/liposome complexes (500–700 nm for FM1-84, FM1-43, SynaptoGreen C3, and FM2-10; 550–750 nm for FM4-64 and FM5-95; and 500–650 nm for SGC5) are indicated by solid squares. Fluorescence intensities of samples that contained dyes but lacked liposomes are indicated by open squares. (I) Dye/liposome fluorescence intensities were corrected by subtracting the free dye signals; these data are plotted versus [dye]. (J) Ratio of fluorescence intensities of dyes with and without liposomes. Error bars are the mean \pm SE from three separate experiments.

FM1-43, SynaptoGreen C3, and FM2-10. This rank order is consistent with the steady-state experiments shown in Fig. 3, and is also consistent with the relative hydrophobicity of each dye: longer and thus more hydrophobic tails result in higher-affinity binding.

For rapid departioning experiments, dyes (4 μ M) were premixed with liposomes (2.2 nM; 30% PE/70% PC) in a small syringe of the stopped-flow spectrometer and then rapidly mixed with buffer from a large syringe, resulting in a 1:11 dilution. Averaged dilution traces are shown in Fig. 6, where the fluorescence signals dropped to a new baseline within several to tens of milliseconds. These rapid fluorescence changes were fitted by single exponential functions (Table 1) as described in Materials and Methods. FM1-84 dissociated from membranes with the slowest kinetics, whereas FM2-10 departed with the fastest kinetics. These differences are due to the different tail lengths of these dyes, as they share the same fluorophore core structure. The new dye, SGC5,

exhibited an intermediate rate of departioning. In rapid departioning experiments, $k_{\text{off}} \gg k_{\text{on}}$, so $k_{\text{diss}} \approx k_{\text{off}}$, and comparisons between the k_{off} and k_{diss} (Table 1) data generated here reveal good overall agreement (the k_{diss} values are somewhat slower than k_{off} when k_{on} is neglected).

To quantify the effect of temperature on dye departioning kinetics, we picked the three brightest dyes—FM1-43, FM1-84, and SGC5—and performed rapid dilution experiments at different temperatures (from 6°C to 37°C; Fig. 7 A). Moreover, because there is a pH gradient across the vesicle membrane in cells, we assayed the rates of departioning of these three dyes at different pH values (from 5.4 to 7.4), once again using a rapid mixing stopped-flow approach (Fig. 7 B). As expected, the departioning kinetics became faster at higher temperature, but the rank order of rates remained constant (Fig. 7 A). However, the kinetics of departioning was unaffected by pH (Fig. 7 B); hence, differences in vesicle lumen pH in living cells is unlikely to

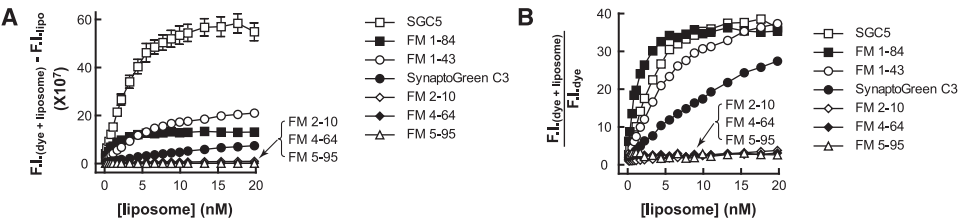


FIGURE 3 Fluorescence intensity of dye-membrane complexes as a function of liposome concentration. (A) Increase in the fluorescence intensity of a fixed concentration of each dye as a function of increasing [liposome] (30% PE/70% PC); data were corrected by subtracting signals obtained from liposome-alone samples. EC₅₀ values, which approximate the affinities of the dyes for

membranes, were determined for the three brightest dyes (SGC5, FM1-84, and FM1-43; EC₅₀ = 4.0, 1.3, and 4.2 nM, respectively). These values are in reasonable agreement with the dissociation constants determined from the kinetic experiments described in Fig. 5 and Table 1. (B) Ratio of fluorescence intensities of all the dyes tested; SGC5, FM1-84, and FM1-43 yielded the highest signal/noise ratios. Error bars are mean ± SE from three independent experiments.

confound interpretation of FM destaining experiments using neurons.

Finally, we tested the influence of lipid composition on the departitioning kinetics of FM1-43 and FM1-84. For these experiments, we generated liposomes composed of total brain lipid or 30% PE/70% PC with increasing amounts of cholesterol to systematically influence membrane fluidity. We note that the molar ratio of cholesterol/phospholipid in synaptic vesicles and synaptosomal membranes is ~45% of the total lipid (42,43). Therefore, we included 5%, 15%, and 45% cholesterol (cholesterol/phospholipid) in PE/PC vesicles. In all cases, the departitioning kinetics of FM1-84 was slower compared with that of FM1-43. Inclusion of cholesterol did not affect the departitioning kinetics of either dye. FM1-43 and FM1-84 dissociated more slowly from vesicles made from total lipid extract derived from brain, but these rates still remained on the millisecond timescale (Fig. 7, C and D).

DISCUSSION

In the work presented here, we compared the biophysical properties of all members of the FM dye family to gain a better understanding of how these probes interact with membranes. We first monitored fluorescence changes as a function of the [dye] and [liposome] to help determine the optimal concentrations of each dye for use in membrane-staining experiments, and to gain qualitative insights into the

relative affinity of each dye. This analysis included a new dye, SGC5, which engages membranes in a manner akin to the FM dyes (Table 1) and becomes highly fluorescent upon insertion into lipid bilayers (Figs. 2, H–J, and 3 A). These experiments revealed that FM1-43, FM1-84, and SGC5 had the highest fluorescence intensities (Figs. 2 I and 3 A) and the greatest signal/noise ratios (Figs. 2 J and 3 B). Hence, the dyes in this subgroup are the most promising candidates for studying single synaptic vesicles (7,9,10,34,44).

We also measured the emission spectra of each dye after loading them into presynaptic nerve terminals (Fig. 4 A), and found that these spectra differ from the spectra obtained using artificial liposomes, as well as the spectra provided by the vendor. However, these experiments confirmed that the emission spectra of FM4-64 and FM5-95 exhibited a significant red shift as compared with other dyes (Fig. 4 A). The distinct red-shifted spectra of these two dyes make them particularly useful for two-color imaging studies, in conjunction with Qdots (45), or green fluorescent protein (33,46–48).

In addition to the steady-state characterization of the dyes, we also determined the kinetics of dye-membrane binding and unbinding reactions using a stopped-flow rapid mixing approach (Figs. 5 A and 6 A). This approach is more sensitive to changes in fluorescence intensity as compared to cell-based experiments, and has the advantage of a much higher time resolution (dead time ~1 ms). From our time-resolved dye-membrane association experiments,

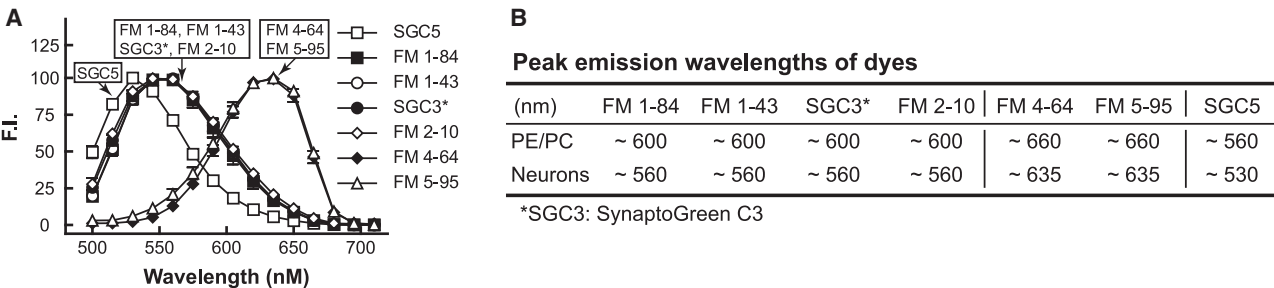


FIGURE 4 Spectra of dyes after loading into hippocampal neurons. (A) Emission spectra from hippocampal neurons loaded with 2 μM SGC5 (open squares), 2 μM FM1-84 (solid squares), 10 μM FM1-43 (open circles), 20 μM SynaptoGreen C3 (solid circles), 40 μM FM2-10 (open diamonds), 10 μM FM4-64 (solid diamonds), or 40 μM FM5-95 (open triangles) were background-corrected and normalized. Error bars are standard deviations (n = 27). (B) Peak emission wavelengths of dyes bound to liposomes (30% PE/70% PC) or loaded into cultured hippocampal neurons.

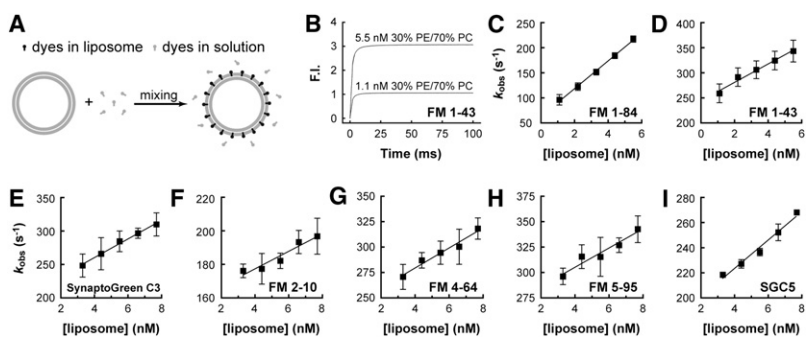


FIGURE 5 Binding and unbinding kinetics of dye-membrane interactions. (A) Outline of the experimental procedure used to measure the association of dyes with membranes. Dye and liposomes (30% PE/70% PC) were rapidly mixed in a stopped-flow spectrometer at a 1:1 volume ratio; binding results in a marked increase in fluorescence intensity. (B) Representative traces of the fluorescence increases that occur when dyes are mixed with two concentrations of liposomes. The traces were well fitted using single exponential functions, and the rate, k_{obs} , was determined. (C–I) The observed rates, k_{obs} , are plotted as a function of [liposome]. The rate constants, k_{on} and k_{off} , were obtained from these plots by assuming pseudo first-order kinetics as detailed in Materials and Methods. The dissociation constants, K_d , were determined from $k_{\text{off}}/k_{\text{on}}$ (Table 1). Error bars represent the mean \pm SE from three independent determinations.

we obtained k_{on} and k_{off} for each dye, and we used these values to calculate K_d for binding (Table 1). For the four dyes that have identical fluorophore cores, the relative affinities (from high to low) were as follows: FM1-84, FM1-43, SynaptoGreen C3, and FM2-10. The on-rates (k_{on}) were similar for all four of these dyes, but the off-rate (k_{off}) for FM1-84 was significantly slower as compared to the other three dyes, whose k_{off} values were in a similar range. It may be possible that, in comparison with the shorter tails of the other dyes, the five-carbon tails of FM1-84 may reach a critical length that results in significantly greater hydrophobic interaction between the dye and lipids such that there is a greater energy barrier for FM1-84 to overcome during departioning from membranes (Fig. 1). This finding

is further supported by the k_{diss} data (Table 1) demonstrating that FM1-84 dissociates from membranes with slower kinetics than the other dyes that possess the same core structure.

Rapid departioning experiments revealed that all of the FM dyes, as well as SGC5, dissociate from membranes on the milliseconds timescale (Table 1), which is much faster than previously reported in studies using live cell perfusion assays (timescale of seconds) (8,49,50). We note that in rapid departioning experiments, $k_{\text{off}} \gg k_{\text{on}}$, so $k_{\text{diss}} \approx k_{\text{off}}$, and the differences between k_{off} and k_{diss} increase with larger k_{on} values (e.g., FM1-84 and FM1-43) (Table 1). Thus, the measurements of both k_{off} and k_{diss} reported here support the conclusion that FM dyes and SGC5 unbind from

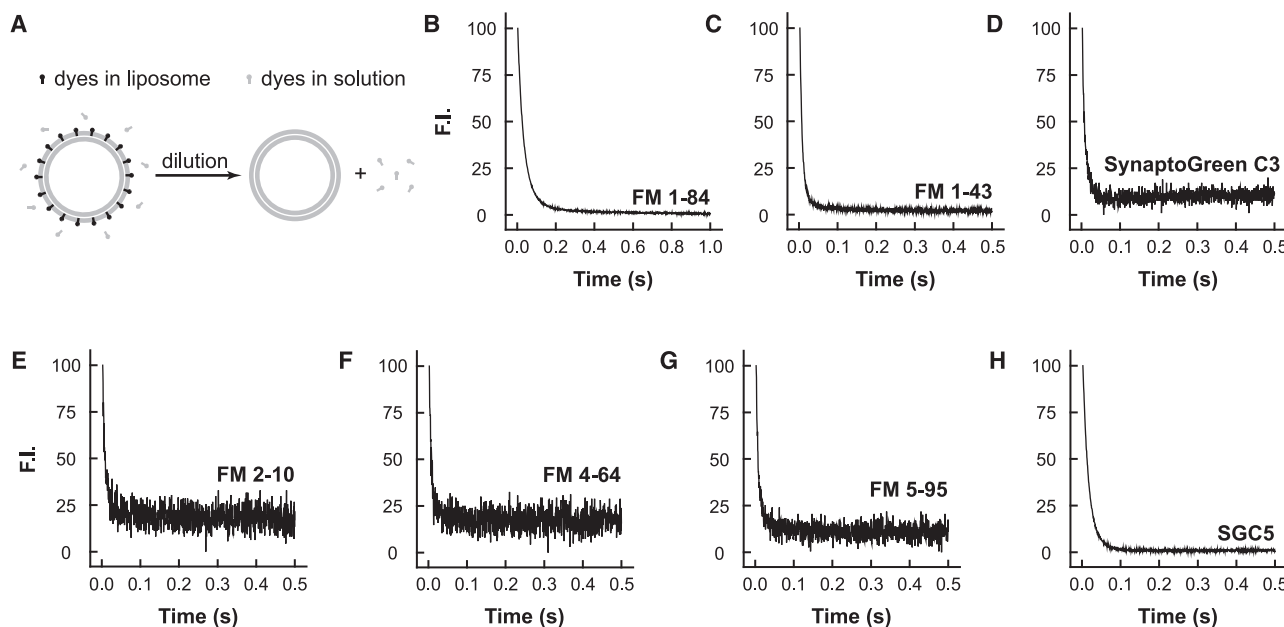


FIGURE 6 Departitioning kinetics of dye-membrane complexes. (A) Outline of the experimental procedure used to measure departitioning kinetics. Dye/liposome mixtures were rapidly diluted (1:11); as membrane-bound dyes dissociated, the fluorescence intensity decreased. (B–H) Representative traces and exponential fits showing the rate of dye dissociation from membranes.

TABLE 1 Kinetics of dye association and departioning with liposomes (30% PE/70% PC, diameter: 100 nm, pH 7.4, 22.0°C)

	FM1-84	FM1-43	SynaptoGreen C3	FM2-10	FM4-64	FM5-95	SGC5
k_{on} ($\text{nM}^{-1} \text{s}^{-1}$)	28 ± 2	18 ± 5	14 ± 4	5 ± 2	10 ± 3	9 ± 3	11 ± 1
k_{off} (s^{-1})	63 ± 7	245 ± 18	204 ± 25	157 ± 30	240 ± 32	251 ± 28	178 ± 6
K_d (nM)	2.3	13.3	14.6	30.0	24.3	26.5	15.8
k_{diss} (s^{-1})	28 ± 1	126 ± 9	149 ± 22	174 ± 35	129 ± 20	131 ± 18	64 ± 2
τ_{diss} (ms)	36.3 ± 0.9	8.0 ± 0.6	7.0 ± 0.9	6.4 ± 1.8	8.1 ± 1.2	7.9 ± 1.0	15.5 ± 0.4

membranes with much faster kinetics than previously thought.

As expected, we found that the rate of dye departioning from membranes became faster at higher temperatures (Fig. 7 A), but not when we varied the membrane fluidity by changing the level of cholesterol (Fig. 7, C and D). These findings provide support for the notion that membrane composition has limited effects on the kinetics of dye binding and unbinding reactions (7). We did observe a ~2-fold reduction in the rate of dye departioning when vesicles were composed of total brain lipid versus vesicles composed of the two major phospholipids in cells (PE and PC; Fig. 7, C and D); however, departioning was still rapid. It is possible that the +2 charge in the dye “head” participates in electrostatic interactions that retard dissociation from membranes harboring anionic phospholipids. Finally, the rates of departioning appear to be independent of pH; similar rates of dissociation were observed from pH 5.4 to pH 7.4. These data suggest that differences in intravesicular pH are unlikely to confound the analysis of single-vesicle destaining data (Fig. 7 B).

The data reported here and previously by Richards et al. (7) demonstrate that the kinetics of FM dye departioning from membranes (on the scale of milliseconds; Table 1) are orders of magnitude faster than the overall destaining rates from presynaptic nerve terminals (on the scale of seconds) (7,8,49,51). Synaptic vesicles labeled with dyes via prior exo- and endocytosis must transit through multiple steps before undergoing rerelease, and these kinetic differences reflect, in part, these processes. Other factors also affect the

rate at which dyes are lost from labeled nerve terminals during exocytosis, including rebinding to the plasma membrane after release and trapping of dyes in synaptic structures. In addition, it is possible that some events occur via nondilating kiss-and-run fusion pores that retard the rate of dye efflux (7). Related to this point are the findings that different FM dyes (e.g., FM1-84, FM1-43, and FM2-10) are released from presynaptic boutons at different rates (8) (but see also Ryan et al. (49), Richards et al. (50), and Fernandez-Alfonso and Ryan (51)). Since, as shown here, these dyes leave membranes with similar rapid kinetics, why do they destain from boutons with distinct kinetics? This puzzle points back to the notion that some release events are mediated by nanometer-scale pores. Since the axial diameter of the FM dyes are similar to the diameter of fusion pores that have been measured directly in a variety of cell types that secrete hormones, transmitters, and other factors (12,15,16,20,21,24–27,29,52–55), small changes in the diameter of the dyes could have significant effects on the rate of efflux through small, nondilating fusion pores. Studies focusing on the dimensions of these dyes and the rates at which they flux through defined pores in membranes (7), rather than their membrane departioning kinetics, are under way and should shed light on these questions.

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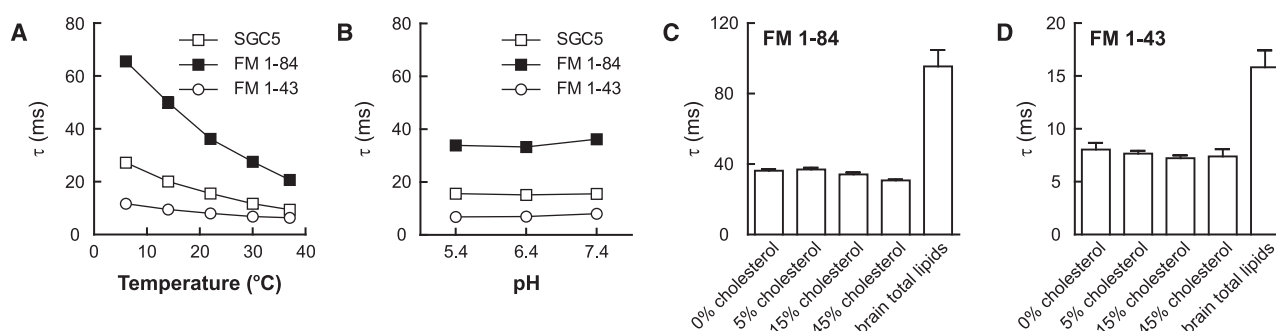


FIGURE 7 Departioning kinetics of dye-membrane complexes as a function of temperature (A), pH (B), and vesicle membrane composition (C and D). In panels C and D, the vesicle composition was 30% PE/70% PC with increasing amounts of cholesterol (cholesterol/phospholipid ratios: 0%, 5%, 15%, and 45%). Departioning was monitored by dilution in a stopped-flow spectrometer; the fluorescence decreases were fit with single exponential functions, and the time constants were plotted.

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